Characterization of Cotton *GhMATE* and *GhALMT1* Systems to Enhance the Acid Soil Tolerance of Cotton Plants (*Gossypium hirsutum* L.)

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Summary of work

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1. Introduction:

Accumulation of high Al^{3+} and protons (H^{+}) in the soil increases acid soil toxicity, promptly affects plant roots architecture, growth, and eventually causes plant death. Al^{3+} ions bind to DNA and RNA molecules and inhibit RNA synthesis (Matsumoto et al. 1977). Plants adapt disparate tolerant and survival mechanisms to become accustomed to various environmental stress factors (Gasper et al. 2002; Kochian et al. 2004). Besides the usual biotic and abiotic stress factors, the acid soil with trivalent Aluminum (Al^{3+}) in solubilized ionic form is the major toxin encountered by plants. About 30 - 40% of world cultivable land is classified under acid soil (Von Uexkuell and Mutert 1995; Duncan 2000). Al^{3+} toxicity is considered as a primary limiting factor for crop production under acid soil conditions. The first and most significant toxicity symptom of Al toxicity is inhibition and suppression of root growth. Furthermore, Al limits the ability of roots to scavenge for nutrients and resulting in a poorly developed root system and reduced biomass. Besides, Al can interact with multiple sites of the cell, including the cell wall, cell membrane and cell cytosol with consequent toxic effects (Rout et al. 2001). Molecular characterization of acid soil stress tolerance mechanism is critical to develop efficient breeding materials to make high yielding varieties under stress environments. Numbers of genes and transcription factors which are liable for Al tolerance have been isolated from various plant species. Initially, genes encoding malate (ALMT1; *Al-activated Malate Transporters*) and citrate transporters (MATE*; Multidrug and Toxic Compound Extrusion*) were recognized as key regulators in the Al-exclusion mechanism (Sasaki et al. 2004; Magalhaes et al. 2007). ALS3 (*Aluminum Sensitive 3*), encoding a half type ATP-binding cassette transporter that may be concerned in the redistribution and sequestration process of Al from sensitive tissues in Arabidopsis (Koyama et al. 1995; Kinraide, 2003; Watanabe and Okada, 2005; Larsen et al.2005).

Rhizotoxicity studies indicate that patterns of damage caused by H^+ are distinct from those induced by Al (Koyama et al. 1995). Additionally, these stress factors were notable by a modeling of root elongation in acidic forest soil (Kinraide, 2003). These reports suggest that H^+ and Al rhizotoxicities induce damage in the roots by different processes. However, several studies selecting Al-tolerant cultivars suggested that H^+ tolerance and Al tolerance are genetically associated in some plant species (Rangel et al. 2005). Besides, large amounts of H^+ ions have adverse effects on the availability of soil nutrients to plants (Kidd and Proctor, 2001). In Particular, under low pH conditions, the solubility of Al and other heavy metal elements increases. Excessive amounts of soluble metal ions are toxic to plants in several ways (Ginocchio et al. 2009). These propose that, although the mechanisms of the toxicities of H^+ and Al would involve distinct biological processes, tolerance to these stresses might be concomitantly regulated in some plant species, possibly by the same genetic factors. The Genes encoding malate (Al-activated malate transporters - *ALMT*) and citrate transporters (Multidrug and toxic compound extrusion – MATE) were well characterized as key genes involved in the Al^{3+}-exclusion mechanism (Hoekenga et al. 2006; Magalhaes et al. 2007; Sawaki et al. 2009) in Arabidopsis and few crop plants. Malate and citrate are the most common organic acids released from roots and are involved in Al^{3+} detoxification mechanism (Kochian et al. 2005). Citrate released from the roots has been demonstrated to be a better chelator for Al^{3+} than malate (Delhaize et al. 1993; Ryan et al. 2001), as the three carboxyl groups of citrate more efficiently detoxifying the Al^{3+} than malate which is having only two carboxyl groups. Based on the above hypothesis, we planned to characterize cotton *ALMT1* and *MATE* systems to develop Al^{3+} stress tolerant cotton plants.

2. Objectives of the Study

I. Cloning and expression profile characterization of *GhALMT1* and *GhMATE* systems by using cotton seedlings.

II. Development of transgenic Arabidopsis plants by using transforming pBI121::35S::*GhMATE* or *GhALMT1* construct.

III. Development of transgenic cotton cell cultures and/or plants by using pBI121::35S::RNAi-*GhMATE* or *GhALMT1* construct.

IV. Characterization of RNAi-*GhMATE* or RNAi-*GhALMT1*, T1 plants by using RT-PCR to study the expression profile of *GhALMT1*, *GhMATE, GhSTOP1*, *GhALS3*, *GhSTAR* and other genes involved in GABA shunt.

V. Relative root growth (RRG) assay and citrate and/or malate estimation will be performed by using Arabidopsis plants over-expressing *GhALMT1* or *GhMATE* with Arabidopsis mutants lines of *AtALMT1* or *AtMATE* or *AtALS3* or *AtSTOP1* or *AtSTAR*

3. Methodology

3.1. Plant Materials

Acid-delinted Cotton (*Gossypiumhirsutum* L. CV. Narashimha) seeds were acquired from Regional Agricultural Research Station, Acharya NG Ranga Agricultural University, Nandyal, Andhra Pradesh, India. *Arabidopsis thaliana* seeds were obtained from the RIKEN BioResource Center (RIKEN BRC). All of the experiments were carried out in an environmentally controlled growth room under 14 h 24°C : 10 h 22°C, light : dark, a light intensity of 200 μmol photons m^{-2} s^{-1} and 70% relative humidity.

3.2. Isolation of cDNA

The total RNA was isolated (RNA isolation kit, Nucleospin, Takara) with oligo (dT) primers. cDNA synthesis was performed with the help of PrimeScript^{TM} 1st strand cDNA synthesis kit (Takara). Full-length *GhMATE* cDNA was obtained by a degenerate PCR and RACE (Rapid Amplification of cDNA Ends) methods (Sawaki et al. 2014; for degenerate primer).The gene-specific primers for 3'-RACE and 5'-RACE were designed by following the sequence of degenerate PCR products. The amplified cDNA fragments were cloned in the pMD20-T clone vector (Takara) and sequenced. The isolated cDNA of *GhMATE1* was deposited into GenBank (accession numbers: MG780413). By following *AtMATE(At1g51340)* and *AtALMT1*(*At1g08430*) CDS sequence, the *MATE* and *ALMT1* orthologous genes were identified by searches of GenBank (www.ncbi.nlm.nih.gov/genbank/) and National Institute of Basic Biology PHYSCObase (http://moss.nibb.ac.jp/). Sequence analysis, amino acid alignment, and phylogenetic tree analysis were carried out using CLC sequence viewer software version 8.0 (Qiagen, Germantown, MD, USA).

3.3. RNAi Vector Construction

The GhMATE-RNAi vector was prepared by replacing the *GUS* gene of the binary vector pBI121 (Toyobo) with the *GhMATE-RNAi* sequence. The sense (3'end) and antisense (5'end) of *GhMATE* sequence consisted of the partial cDNA (311 bp; 1102 to 1442) joined to first intron of the Arabidopsis isocitrate dehydrogenase (*At1g65930*) sequence (Nakagawa et al. 2007). The *GhMATE-RNAi* cassette was under the control of

cauliflower mosaic virus (CaMV) 35S promoter, while NPTII gene was under the control of NOS promoter. After the confirmation of gene orientation by sequence analysis, the pBI121-GhMATE-RNAi vector and control vector (pBI121) were introduced into *A. tumefaciens* (EHA105) by the freeze–thaw method and then into cotton by seed embryo apex explants transformation.

3.4. Cotton Transformation

For cotton transformation, we used seed embryo apex explants. The surface sterilized cotton seeds (70% ethanol for 1 min and $HgCl_2$ for 4 mins) were soaked in sterile distilled water for 2 days in the dark. The seeds with opened seed coat were selected for embryo apex rescue (Meristemal region). The explants were cultured in upright position MS medium fortified with B5 vitamins, 3% glucose, 0.8% agar and (2.0 mg/L) N-6-benzylaminopurine (BAP). The excised meristematic apex further selected for transformation studies. The transformed *A. tumefaciens* cells were harvested and resuspended in 10mL liquid infection media (calcium-free MS basal medium supplemented with 1.0 mg/L BAP, 10 g/L glucose, 20 g/L sucrose 100 mg/L acetosyringone, 0.02 % pluronic F68, pH 5.2). Embryo apex explants were then immersed in the bacterial suspension for 20 min. with simple agitation. Following a quick drying on the sterile filter paper, they were co-cultivated on a co-culture medium (calcium-free MS basal medium supplemented with 1.0 mg/L BAP, 10 g/L glucose, 20 g/L sucrose, 100mg/L acetosyringone, 2 g/L gelrite) at 28°C in the dark. After 5-6 days of co-cultivation, the calli were washed with sterile water containing 500 mg/L carbenicillin, and transferred to the MS basal medium supplemented with 2 mg/L BA, 30 g/L glucose, 2 g/L gelrite, 250 mg/L carbenicillin and 100 mg/L Kanamycin for pre-selection. The cultures were maintained in 16h per day light conditions and temperature of 25°C ± 2°C and sub cultured every week. After 28 days of culture, the healthy shoots (min 2.0 cm in height) were excised from the multiple shoot cultures and transferred individually to root induction media consists of MS basal salts along with B5 vitamins, 0.1 mg/l Indole Butric Acid (IBA), 30 g/L glucose and 100 mg/L Kanamycin (Ganesan et al. 2009; Pathi and Tuteja 2013). After 28 days of culture, the plantlets with well-developed roots (min 4 cm in length) were hardened and maintained for 3 weeks in the culture room. Plantlets with the hardened roots were transferred to small pots containing

a mixture of vermiculite and sand in 1:1 ratio and maintained under high humidity conditions. Throughout the paper, the *GhMATE-RNAi* lines were designated as KD.

3.5. Overexpression Vector Construction and Arabidopsis Transformation

The *GhMATE* overexpression vector was prepared by replacing the *GUS* gene of the binary vector pBI121 (Toyobo) with the *GhMATE* sequence. The binary vector pBI121 carrying cotton *GhMATE1* under the control of CaMV35S promoter while NPTII gene was under the control of NOS promoter. After the confirmation of gene orientation by sequence analysis, was introduced into *Agrobacterium tumefaciens* strain EHA105 and transformed into WT Arabidopsis plants (Col 0) by the floral dip method (Clough and Bent, 1998).

3.6. Molecular analysis of transgenic plants and seed collection

PCR positive transgenic plants were considered for molecular confirmation. The genomic DNA was isolated from young leaves of transformed (T0 generation) and wild-type (WT) cotton plants using PureLink® PlantTotal DNA Purification kit from Invitrogen. For all transgenic plant lines, T1 seeds were collected from PCR positive transgenic plants (T0) were planted on half-strength Murashige and Skoog medium supplemented with 0.5 mg/L BAP, 0.5 mg/L GA_3 and 60 mg/L Kanamycin. The PCR mixture was prepared and performed with PrimeSTAR Max DNA Polymerase (R045B, Takara, Japan). PCR amplifications were performed at the following conditions: 92˚C for 4 min; followed by 30 cycles of 92˚C for 1 min; 10 cycles of 60°C (-0.5˚C step-down) for 15 sec, 26 cycles of 55˚C 15 sec, 72˚C for 30 sec; and a last elongation step of 72˚C for 3 min. The amplicon obtained had 350 bp. The PCR positive transgenic plants were transferred to plastic pots containing soil and vermiculite with the ratio of 1:1 (w/w) in room temperature (25^{o}C) with 16 h light/day to collect T1 seeds. The T1 transgenic seeds were collected and transgenic seedlings were selected on MS (Murashige and Skoog, 1962) selection media containing MS salts, B5 Vitamin, 30 g/L glucose, 100 mg/L Kanaymyin, 8.0 g/L agar at pH 5.8. 3 days old, Kanamycin tolerant transgenic seedlings were considered for further experiment. The suppressed lines were designated as GhMATE1-KD (Knock-Down). For Southern-blot analysis, genomic DNA was isolated from the leaves of greenhouse-grown plants and digested with HindIII. DNA (20 µg/lane)

was separated by using 0.8 % agarose gel electrophoresis and transferred to Amersham Hybond XL membranes (GE Healthcare). Southern hybridization was performed using a non-radioactive DNA labeling and detection protocol.

3.7. Hydroponic Culture and Stress Treatments

The WT and transgenic cotton seeds were grown aseptically. The germinated seeds of each line were placed on a polystyrene float on culture solution. The basal solution lacked inorganic phosphorus and contained 2% (w/v) MGRL nutrients and 200 mM $CaCl_2$ (Kobayashi et al. 2007). The H^+ and Al^{3+} toxic solutions were prepared by adjusting the pH with HCl and adding $AlCl_3$ to obtain concentrations known to be toxic to cotton seedlings (pH 4.4 for H^+ and pH 4.75+20 µM for Al^{3+} toxicity). Culture solution at pH 5.5 without Al served as the control to evaluate Al^{3+} and H^+ toxicity. For *Arabidopsis* experiments, surface-sterilized seeds (75% ethanol for 4 min), were stratified at 4°C for 3 days and planted on Tissue culture containers (PW1301, Himedia, India) which contains 1/5 Hoagland nutrient solution supplemented with 1 mM MES and 0.8% agar (pH 5.5). After 7 days, the healthy seedlings with uniform size were selected for Al stress studies. All solutions were renewed every 2d, and seedlings were grown for 15-18 d at 25°C. After collecting the T1 seeds, five to eight independent RNAi lines were selected for each treatment.

3.8. Expression Analysis

Real-time RT-PCR for quantitative analysis was carried out using Power SYBR Green (Applied Biosystems) and an ABI PRISM 7000 instrument (Applied Biosystems) according to the manufacturer's protocol for the standard curve method. For all experiments, a diluted cDNA series was used to obtain standard curves of target genes and the internal control.

3.9. Morphological Characterization of Transgenic Plants

For root architecture analysis, roots were placed on graph sheets and analyzed (Obiefuna and Ndubizu 1979). With the help of ImageJ software, the top 7 lateral roots were considered to calculate the mean value of lateral root. To observe the variations in

secondary root growth, 7d or 14 d old seedlings were treated with low pH and high aluminum stress conditions. After 7 more days of growth, the length of the primary root was measured to the nearest millimeter with a ruler. The number of lateral roots on each primary root was determined by counting all lateral roots that had emerged from the primary root (Rashotte et al. 2001). The variation in root growth pattern was also recorded by using ImageJ software.

3.10. Collection of Root Exudates, and Quantification of Citrate

Cotton seedlings were grown for 7 days in 100 mL of 1/50 MGRL nutrient with 200 mM $CaCl_2$ solution containing 1% Sucrose under aseptic conditions (pH 5.5) in plastic pots. For stress treatment selected lines were treated with 20µM $AlCl_3$ (pH 4.75) or low pH stress (4.4). Citrate was quantified by the enzyme reaction (citrate lyase for citrate)-coupled NADH/NAD+ cycling method with minor modification (Hampp et al. 1984).

3.11. Hematoxylin Staining

Cotton seedlings were grown for 7 days in 100 mL of 1/50 MGRL nutrient with 200 mM $CaCl_2$ solution containing 1% Sucrose under aseptic conditions (pH 5.5) in plastic pots. For stress treatment selected lines were treated with 20µM $AlCl_3$ (pH 4.75) for 5mins, then dipped in 0.2 % iron hematoxylin and 0.02 KIO3 for 5 mins (Polle et al. 1978). The excess dye was eliminated by washing the roots in deionized water under aeration for 15 mins.

3.12. Statistical Analyses

Means and standard errors were used throughout the study and the values were assessed using a parametric moods median test (n = 5-15) (Snedecor and Cochran 1989). The data were analyzed for variance by Duncan's multiple range test (DMRT) using the SPSS statistical programme version 7.0 in necessary places.

4.0 RESULTS

4.1. Characterization of GhMATE-RNAi plants under acid soil stress conditions

MATE family alone contributes to the Al resistance in plants *via* organic anion efflux (citric acid) in few species. Moreover, the *MATE* gene expression was activated only under Al^{3+} stress conditions (Liu et al. 2009). Besides, our preliminary results showed that the cotton seedlings exhibited sensitive phenotypes under Al and H^+ toxicities. Under low pH conditions, in particular, pH 4.4 treatment displayed root growth inhibition in cotton seedlings. On the other hand, 20µM Al treatment at pH 4.75 showed retarded primary and secondary root growth development. Based on the above observations, we targeted to characterize the *GhMATE* system which is one of the key regulators of Al exclusion mechanisms. By following standard procedures, we amplified the full length of cotton *GhMATE*. The functional cDNA sequence of cotton MATE (*GhMATE*) consists of 1551bp which encodes 552 aa residues. Phylogenetic analysis indicated that *GhMATE* is more closely related with other Malvaceae members.

We developed transgenic cotton plants with GhMATE-RNAi construct by *Agrobacterium tumefaciens* -mediated transformation by infecting the embryo apex explants (Ganesan et al. 2009). The kanamycin tolerance putative transgenic plants were further selected for PCR confirmation. The T1 seeds were collected from the PCR positive T0 transgenic plants were allowed for self-pollination. Furthermore, Kanamycin tolerant 3d old seedlings (KD lines) were selected for Al stress tolerance studies. Under Al and proton stress conditions, transgenic plants showed repressed expression of *GhMATE* and displayed very sensitive phenotypes. The stress assay was made for long time exposure under hydroponic conditions. All the seedlings were treated for 7 days and compared with the control plants [WT and randomly selected vector control (VC) plants].

Under control conditions (pH 4.75), all KD lines showed no significant root growth variations when compared with control plants. Besides, under Al (pH 4.75 + 20µM) and proton (pH 4.4) stress conditions, we observed significant decline in the primary and secondary root length of RNAi transgenic lines. Al sensitivity was significantly enhanced in *GhMATE1* down-regulated lines. After 7 days' treatment, under

Al stress conditions (pH 4.75 + 20μM), the transgenic lines exhibited 27% inhibition in the primary root length when compared with control plants. Moreover, the primary root growth was ~62% inhibited for RNAi plants under proton toxicity (pH 4.4) treatments. Besides, we also observed retarded secondary root initiation and growth process in KD plants under Al rhizotoxicity conditions. For the secondary root initiation studies, all the seedlings (control and transgenics) were maintained under control conditions (pH 5.5) for 4 days. For Al stress treatments, the seedlings were transferred to different stress conditions. After 12 days' growth, we also observed suppressed secondary root growth between RNAi transgenic and WT plants under Al and proton toxic conditions. In particular, Al stress displayed ~32% decline in the secondary root length of *GhMATE* suppressed lines than in control plants. On the other hand, under proton stress conditions also the root length of *GhMATE-RNAi* seedlings was inhibited (~11 %) than in control plants. These results suggest that *GhMATE* is necessary for both H^+ and Al tolerance mechanisms by regulating the primary and secondary root growth of cotton. Moreover, down-regulation of *GhMATE* also suppressed the secondary root initiation process under Al stress conditions (**Fig.1**). Only limited number of reports showed that Al stress also inhibits secondary root growth. In barley, Al toxicity inhibited the growth of newly-emerged lateral roots (Hecht-Buchholz and Foy 1981; Tamas et al. 2003). Besides, the root hair numbers were significantly decreased in Al treated Clover plants (Care 1995).Recently, Wang et al (2017) studied Al^{3+}-induced IAA transport in alfalfa plants and their results indicated that the Al^{3+}-induced reduction of root growth could be associated with the inhibitions of IAA synthesis in apical buds and imbalanced distribution of IAA in root tips.

4.2. Repression of Al-Activated Citrate Excretion and Al Stress Responsive Genes

Under Al stress conditions (pH 4.75, 20 μM for 48h), the wild type plants secreted highest concentration of citrate when compared with untreated control plants. Citrate level was undetectable in Al-untreated WT and *GhMATE-RNAi* plants. Moreover, all RNAi transgenic lines showed suppressed levels (18 %) of Al-inducible citrate than in control plants. These results suggested that the repressed secretion of citrate is one of the key mechanism controlled by *GhMATE*. Several reports proved that Al tolerance is achieved by organic acid release into the rhizhospehere. Miyasaka et al. (1991) showed that an Al-resistant cultivar *Phaseolus vulgaris* excreted eight-fold increase in citrate

excretion than Al-sensitive cultivars. Moreover, overexpression of citrate synthase resulted in enhanced Al resistance (de la Fuente et al. 1997; Koyama et al. 2001 etc.). Moreover, we measured the amount of Al accumulation by using hematoxylin staining. Both RNAi plants and control were treated with Al (20 μM pH 4.75) for 15 mins and the variations in the density of purple color were recorded. The RNAi plants displayed denser purple staining than in control plants which indicate greater Al accumulation. The above result stating that MATE expression is essential to control the Al accumulation in the root system.

In continuation of these results, we studied the variations in the expression profile of different genes which are responsible for Al tolerance mechanisms. We evaluated the expression profile of *GhSTOP1*, *GhALMT1*, and *GhALS3.* The STOP1 (*Sensitivity to Proton rhizotoxicity 1*) is one of the essential transcription factor required for regulation of Al^{3+} and H^{+}rhizotoxicity mechanisms functions by regulating the expression of *AtALMT1*, *AtMATE* and several genes (Iuchi et al. 2007). The transcript levels of all the above genes (*GhSTOP1, GhALMT1 and GhALS3*) were not affected in Al-treated lines and both WT and RNAi lines displayed almost same expression profile under Al stress conditions and this result suggests that the expression of all the above genes are not *GhMATE* dependent. Our studies also demonstrated that *GhMATE* expression is regulated by *GhSTOP1* under Al stress condition (Kundu et al. 2018).

We also studied the impact of Al stress effects in two important GABA shunt genes. In our study, the expression profile of *GABAT* and *GAD* were significantly down-regulated in the KD lines when compared with Al treated control plants. We noticed, approximately 74 and 82% decline in the expression of *GABAT* and *GAD* genes respectively in *GhMATE-RNAi* plants. These results suggest that the *GhMATE* system is necessary to regulate the key enzymes of GABA shunt under Al stress conditions. γ-aminobutyric acid (GABA) is a non-protein amino acid involved in various stress tolerance mechanisms of plants and GABA levels of plants rapidly increased when exposed to different stress factors (Kinnersley and Turano 2000; Barbosa et al. 2010). Under stress conditions, GABA may act as a stress-induced intercellular signaling molecule related to the signal transduction pathway in plants (Bouché and Fromm

2004). Song et al (2010) reported that Exogenous GABA application can alleviate oxidative damage caused by H^+ and Al^{3+}toxicities by activating antioxidant defense responses and by reducing the carbonylated proteins caused by ROS. Besides, genes encoding enzymes in the GABA shunt pathway (*GDH1* and *GAD1*) were down-regulated in the Arabidopsis *stop1* mutant. In addition, *GABA-T* (GABA amino transferase) and *GDH2* tended to be down-regulated in *STOP1*-KO plants. Taken together, we could assume that GABA genes are regulated by STOP1 system under acid soil stress conditions (Sawaki et al. 2009).

4.3. Al^{3+} Effect on *GhNAC1* and *GhFRD3* Expression

NAC proteins are plant-specific transcription factors which are having essential functions in plant growth and abiotic stress responses. NAC-type proteins share a conserved N-terminal DNA-binding domain called a NAC (NAM, ATAF1/2, CUC1) domain (Gunapati et al, 2016). Several *NAC* genes play important roles in auxin signaling and lateral root formation, specifically *NAC1* is involved in auxin signaling pathway and activated by auxin and which directs lateral root formation in Arabidopsis (Benkova and Hejatko 2009; Jung and McCouch 2013). The *GhNAC1* expression was suppressed 45 and 23% in KD plants when compared with Al treated and untreated WT plants respectively. Besides, *GhNAC1* expression displayed 21% decline in the transgenic plants when compared with untreated transgenic plants.

Many studies have revealed that Al-induced inhibition of root growth often related to the abnormal accumulation of phytohormones and overexpression of *AtNAC2* enhanced the lateral root development (Yang et al. 2014; Liu et al. 2016). Under Al stress conditions, the miRNA 164 modulates lateral root development by regulating the transcription factors with NAC domain (*CUC1* [cup-shaped-cotyledon 1], *CUC2*, *NAM*, *NAC1*) (Laufs et al. 2004). In particular, miRNA 164 can induce the degradation of endogenous and transgenic *nac1* mRNA, weaken auxin signal to inhibit lateral root growth (Guo et al. 2005). However, overexpression of miRNA downregulates the expression of *NAC1* gene and reduces the formation of lateral root (Liu et al. 2009).

FRD3 (*Ferric Reductase Deficient 3*) belongs to sub-group of MATE proteins in plants and function as citrate transporters and are also involved in the translocation of iron (Fe) from the roots to the shoots. In our studies, the *GhFRD3* expression significantly decreased in *GhMATE* down-regulated lines when compared with WT plants treated with/without Al. Besides, our results also pointed out that Al treatment showed less variation in the *GhFRD3* expression between Al treated and non-treated plants. These results suggest that FRD3 expression is depends on MATE expression in cotton under Al stress conditions and possibly contributes minimal role in Al stress mediated citrate excretion. The variations in the expression level of *GhFRD3* is very low in WT and transgenic plants under normal and Al stress conditions, but is suppressed significantly by Al in *GhMATE-RNAi* lines. A detailed functional study on *GhFRD3* will provide more information about its role in Al stress responses.

4.4. Overexpression of *GhMATE* in Arabidopsis

Our results highlighted that the *GhMATE*-overexpression in Arabidopsis plants displayed enhanced root growth, under control and Al stress conditions. We also recorded enhanced citrate excretion in transgenic plants than in WT plants. The 10 days old Kanamycin tolerant T3 transgenic seedlings were subjected to Al stress (pH 4.75, 10.0 µM $AlCl_3$) studies. After 3 days of maintenance in hydroponic media, both WT and overexpressor plants were treated with Al (4 days). We noticed significant variations in the seedling development. We noticed approximately 31% decline in the primary root length of WT plants under Al stress condition when compared with WT untreated plants. Besides, *GhMATE* overexpressors displayed significantly enhanced the root growth under Al stress conditions when compared with Al treated WT plants. Moreover, the *GhMATE* transgenic plants exhibited ~16% increase in the primary root growth under control conditions (without Al) (**Fig.2**).

In our earlier studies, we observed that *GhSTOP1* is involved in Al tolerance levels and controls the primary and secondary root growth under Al stress conditions (Kundu et al. 2018). Our present study pointed out that the *GhMATE* is also involved in Al tolerance mechanisms by citrate excretion and by regulating primary and secondary root growth. Similar to our results, Arabidopsis plants overexpressing the maize *MATE* gene (*SbMATE*) displayed significant Al tolerant phenotype by increased root

citrate exudation when compared to WT plants (Megalhaes et al. 2007). In addition, overexpression of a *MATE* gene of *Vigna umbellata* (*VuMATE*) in tomato exhibited Al resistance and enhanced citrate efflux (Yang et al.2011). Recent reports also proved that overexpression of *BdMATE* from *Brachypodium distachyon* exhibited Al tolerance in *Setaria viridis* plants (Riberio et al. 2017).Transgenic Arabidopsis lines, which are Al tolerant, overexpressing *BoMATE* displayed lower K^+ efflux and higher H^+ efflux (Wu et al.2014). These results suggest that *MATE* genes are involved in citrate excretion and regulation of K^+ and H^+ efflux.

5.0 Conclusion

Under acid soil stress conditions (Al ion 10 μM to 100 μM) the crop plants are facing retarded root growth and low yield. Variations in the Al concentrations play an important role in plant species distribution under natural environment conditions. There are several genes and transcription factors are involved in Al detoxification mechanisms. In particular, the MATE transporters have been implicated in detoxification mechanisms of Al and heavy metals, disease resistance, nutrient homeostasis, and the transport of secondary metabolites, and growth hormones (Li et al. 2002; Liu et al. 2016; Wang et al. 2017). Since MATE transporters are involved in several stress and physiological regulations, it is very important to characterize the functions of MATE family genes.

Our studies narrated that *GhMATE* expression is Al stress dependent and necessary for Al tolerance mechanisms by regulating different genes which are responsible for root growth and citrate excretion. Expression profile data of RNAi-*GhMATE* transgenic plants demonstrated that *GhMATE* governs the expression of *GhFRD3* (involved with Fe nutrition and expressed in periderm region), *GhGABA*-T, *GhGAD* (key genes involved in GABA shunt) and *GhNAC1* genes which are involved in root development and citrate excretion. Besides, the expression of *GhMATE* is controlled by *GhSTOP1* (*Sensitivity to Proton rhizotoxicity 1*) system which also controls the expression of *GhALMT1* which is necessary for malate excretion under Al stress conditions (**Fig.3**; Kundu et al. 2018). Our results also pointed out that overexpression of *GhMATE* in Arabidopsis enhances the Al tolerance mechanisms by enhancing the citrate excretion and root growth. Based on these observations, we suggest that *GhMATE* could be useful to develop Al tolerance transgenic crops.

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A list of abbreviations:

ALMT1	Al-activated Malate Transporters
MATE	Multidrug and Toxic Compound Extrusion
ALS3	Aluminum Sensitive 3
STOP1	Sensitivity to Proton rhizotoxicity 1
GABA	γ-aminobutyric acid
GABA-T	GABA aminotransferase
GAD	Glutamic acid decarboxylase
NAC	(NAM, ATAF, and CUC) proteins
CUC1	Cup-shaped-cotyledon 1
FRD3	Ferric Reductase Deficient 3
$AlCl_3$	Aluminum chloride
BAP	6, Benzyl Amino Purine
B5	Gamborg's Medium (1969)
CaMV	Cauliflower mosaic virus
$CaCl_2$	Calcium chloride
Cb	Carbenicillin
°C	Centigrade
cm	Centimeter
cv	Cultivar
DNA	Deoxyribo Nucleic Acid
dNTP's	deoxy-nucleotide triphosphate
DMRT	Duncan's Multiple Range Test
2,4-D	2,4-Dichloro phenoxy acetic acid
EDTA	Ethylene diamine tetra acetate
g/l	gram per litre
HCl	Hydrochloric acid

$HgCl_2$	Mercuric Chloride
hr/hrs	hours
IBA	Indole-3-Butyric acid
Kb	kilo base pair(s)
KD	Knock-Down
kD	kilo Dalton
Kan	Kanamycin
KIO_3	Potassium Iodate
L	Litre
LB	Luria broth
µM	Micro molar
µm	micro meter
mg	Milligram
mm	Millimeter
mM	Millimolar
mL	Milliliter
min	Minutes
M	Molar
MS	Murashige and Skoog's Medium
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
NOS	Nopaline synthase
NPT II	Neomycin phosphor transferase
OD	Optical density
PCR	Polymerase chain reaction
%	Percentage
PGRs	Plant growth regulators

PPT	Phosphinothricin
RACE	Rapid Amplification of cDNA Ends
RH	Relative Humidity
RNase	Ribonuclease
RNA-i	RNA-interference
rpm	rotation per minute
s	Second(s)
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SE	Standard Error
TAE	Tris acetate – EDTA
T-DNA	Transferred DNA
Ti	Tumor - inducing
Tris	Tris (hdoxymethyl)-aminomethane
µg	Microgram
µl	Microliter
µM	Micromolar
v/v	volume/volume
VC	Vector control
WT	Wild type
w/v	Weight / volume

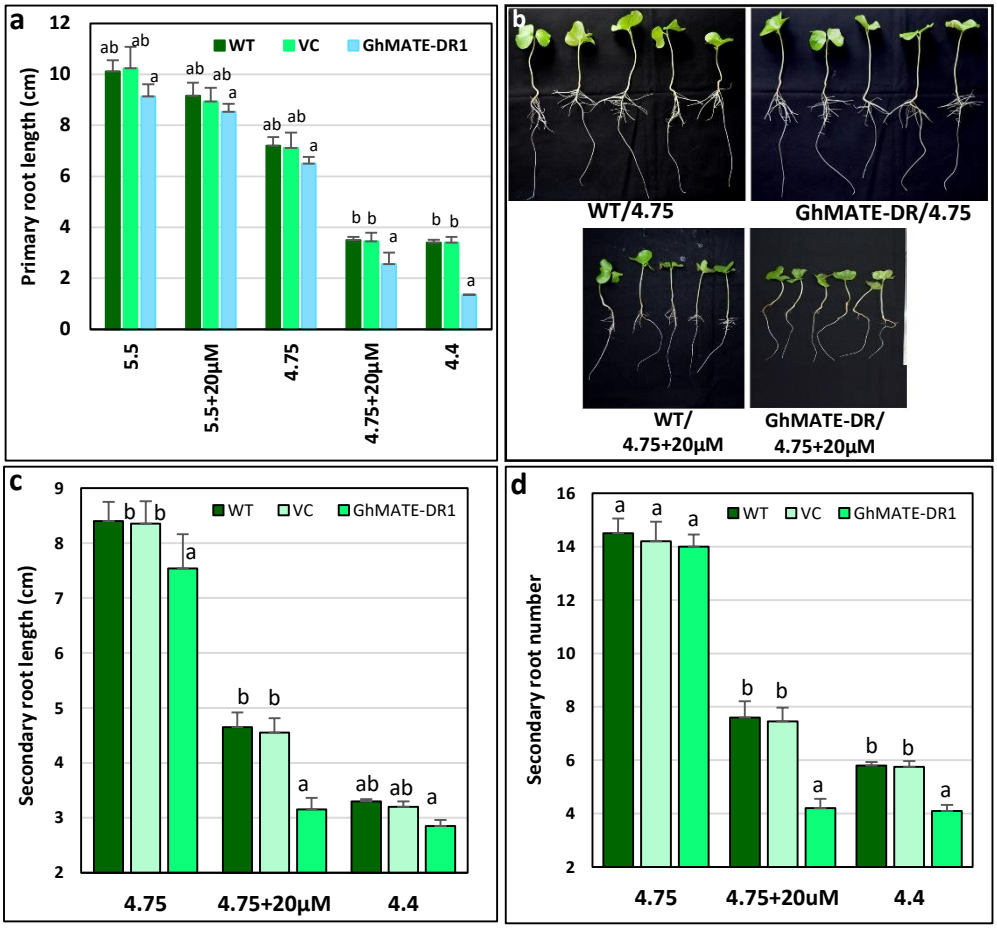

Figure 1. Phenotypic analyses of WT and *GhMATE-RNAi* transgenic seedlings under Al and H^+ stress conditions. Variations in the primary root length (a & b) of 14d old seedlings under different stress conditions. Variations in the secondary root length (c) and root numbers (d) were also calculated from 14d old seedlings. *WT* non-transgenic plants, *VC* transgenic plant with pBI121 (i.e.,"vector only" control), *GhMATE-DR1* two replicates of three randomly selected transgenic lines with *GhMATE-RNAi* construct. (d – f) Al and H^+ stress responsive seedling phenotypes of cotton (12 days old). Each value indicates the mean ± SE (n = 7 for WT and n = 6 for transgenic plants). Mean values within a treatment followed by the same alphabets are not significant at P=0.05 according to DMRT.

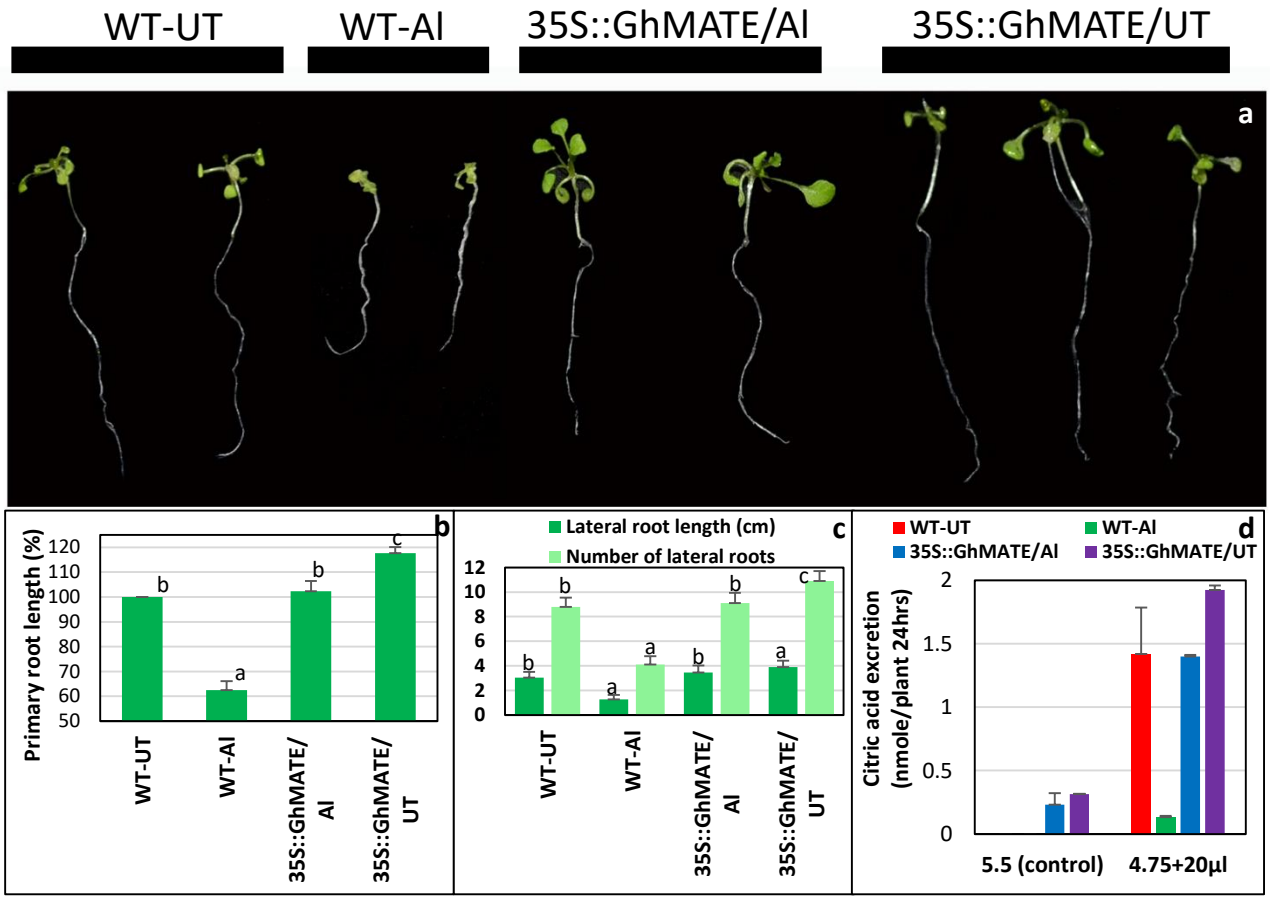

Figure 2. Al induced variations in the phenotypes of *Arabidopsis thaliana* plants 20d old wild type (WT), *MATE* mutant (*AtMATE1*) and transgenic plants (overexpressed with *GhMATE*) (pH 5.5, 20 µM Al treated for 48 h). Primary and secondary root length values (a - c) were calculated after Al treatment. Variations in the citrate release (d) was calculated from the hydropic media. *WT* non-transgenic plants, *VC* transgenic plant with pBI121 (i.e.,"vector only" control), *35S::GhMATE* plants are wild-type Col-0 plants which were transformed with overexpression construct. Each value indicates the mean ± SE (n = 6). Mean values within a treatment followed by the same alphabets are not significant at P=0.05 according to DMRT.

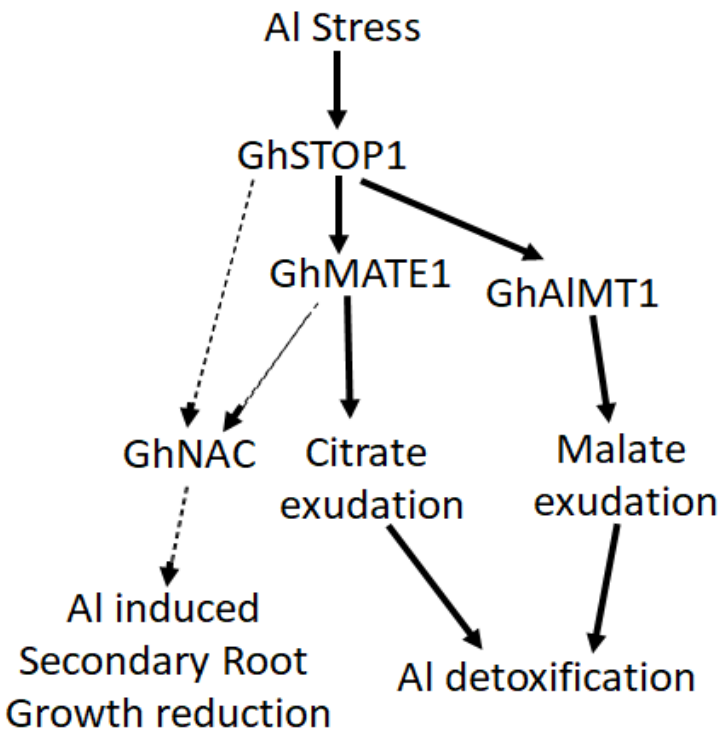

Figure 3. The possible Al detoxification mechanism involved in cotton. Aluminum (Al) activates the GhSTOP1 transcription factor which regulates *GhALMT1* and *GhMATE1* which are involved malate and citrate excretion respectively. The presence of OA in the rhizosphere possibly prevents Al from entering the cell. The expression of *GhNAC* transcription factors are also controlled by *GhSTOP1* system and *GhMATE1* under AL stress condition. Al, aluminum; STOP1, sensitive to proton toxicity 1, ALMT, aluminum-activated malate transporter; MATE, multidrug and toxic compound extrusion family.